



Validated RP-HPLC Method for Quantification of Paclitaxel in Human Plasma – Eliminates Negative Influence of Cremophor El

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ABSTRACT

Background: Literature reports innumerable methods for quantification of paclitaxel in biological matrices. Most of these involve complicated extraction procedures like solid phase extraction, separate procedure for elimination of interference of Cremophor El, advanced and expensive instruments.

Objectives: The objective of the present research work is to develop and validate a simple, rapid, sensitive, economic and reproducible reverse phase – high performance liquid chromatography method for the estimation of paclitaxel concentration in human plasma that eliminates negative influence of Cremophor El on recovery of paclitaxel.

Methods: Chromatographic separation of paclitaxel was carried out using C18 column (150 × 4.6 mm i.d., 4µm particle size, Waters, Australia) with 60% acetonitrile, 40% of 10mM ammonium acetate buffer solution and 0.1% formic acid as a mobile phase at a flow rate of 1.0mL/min at ambient temperature. Validation was performed as per ICH Q2 guidelines.

Results: In this system the retention time was 3min. The detection limit was 5ng/mL and limit of quantification with reproducibility was 15ng/mL. Plasma samples were extracted using single solvent (tertiary – Butyl Methyl Ether) liquid- liquid extraction with a recovery of 96-99%. Robustness of the method was established with variation in flow rate, detection wave length and mobile phase composition. Stability of paclitaxel during the study period was studied and found to be stable.

Conclusion: The developed method is easy to perform, quick, reproducible with good recovery without negative influence of Cremophor El and applicable to quantify paclitaxel in regular clinical practice for individualization of the therapies.

Key Words: Paclitaxel, Cremophor El, Single solvent extraction, Bioanalytical

INTRODUCTION

Paclitaxel is a very effective antineoplastic agent used in the treatment of ovarian, breast, cervix and non-small cell lung cancers (1). Paclitaxel was the first taxane derivative isolated from the bark of *Taxusbrevifolia* in the year 1971 (2) and being marketed from 1993 (3). It is a complex, hydrophobic molecule administered in a solution of Cremophor-EL (Cr El) and dehydrated alcohol in the ratio of 1:1.

Neutropenia, myelosuppression, gastrointestinal ailments, alopecia are the common adverse effects of paclitaxel treatment. Peripheral neuropathy is the major adverse effect of paclitaxel and increases with increasing cumulative dosage (2). Moreover, Cr El is widely known to cause severe hypersensitivity reactions requiring premedication (4).

It is well recognized that there is inter and intra individual variability in pharmacokinetic characteristics of paclitaxel.

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Currently body surface area (BSA) is used in the dosing of paclitaxel, leading to significant inter individual differences in plasma concentrations and risk of severe and treatment limiting adverse effects (5, 6). Quantification of paclitaxel in the blood or plasma samples is necessary to establish pharmacokinetic parameters that can assess inter and intra individual variability, thereby making necessary dose adjustments to improve therapeutic efficacy and minimizing the adverse effects (7). It is also reported that Cr El has a negative impact on the reproducibility of bioanalysis of paclitaxel due to entrapment of paclitaxel in the micelles of Cr El (8), and various methods are used to nullify the effect of Cr El on quantification of paclitaxel (9).

Therefore, it is necessary to develop a rapid, simple, sensitive, economic and reproducible method to quantify plasma concentrations of paclitaxel in regular clinical practice to promote individualization of treatments.

Till date several methods have been reported for the quantification of paclitaxel in biological matrices using various techniques like high performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectroscopy (LC-MS), micellar electrokinetic chromatography (MEKC), capillary electrophoresis, immune assays etc (10-12). Most of these methods require tedious, complicated extraction procedures like solid phase extraction (SPE), advanced and expensive instruments like LC-MS which are not available in many hospitals, diagnostic and research labs.

We herewith report on our development of a rapid, simplified, economic and sensitive method of paclitaxel quantification in human plasma by liquid-liquid extraction for application in clinical sample analysis.

METHODS

Instruments and Reagents

The HPLC system - LC- 2010 (Shimadzu Corporation, Kyoto, Japan) liquid chromatographic quaternary auto sampler pump model equipped with UV-Visible detector, Class VP software. C₁₈ HPLC column with 150×4.6mm (i.d) with particle size of 4μm (Waters India Pvt Ltd), Universal guard column with 20×3.9mm i.d and UV detector (Shimadzu), Ultra sonicator (PCI analytics, Vertex enterprises, India), Milli Q water system, Micro centrifuge and vortex shaker.

Paclitaxel (>99% purity) was purchased from Sigma-Aldrich, India. Paclitaxel formulation (Taxeleon 30mg/5mL) was kindly gifted by Neon Laboratories, Mumbai. Acetonitrile, t-butyl methyl ether (t-BME), methanol of HPLC grade, ammonium acetate and formic acid of analytical grade were used. Drug free plasma was obtained from healthy volunteers in our laboratory.

Preparation of stock solution

Stock solution of paclitaxel was prepared by dissolving 10mg of paclitaxel in 10mL of acetonitrile. This stock solution was further diluted for the preparation of working standard solutions with diluent (Acetonitrile & water in 1:1 ratio) to get the concentration range of 6ng/mL to 6μg/mL.

Blood sampling

This method development is a part of pharmacokinetic drug interaction study in cancer patients and has been approved by Institutional ethics committee of our institution (IEC No: IEC-NI/16/Mar/51/16). Blood samples from six cancer patients receiving 260mg of Paclitaxel were collected after procuring signed written informed consent. All the blood samples were collected in heparinized vacutainers. The plasma samples were separated from the whole blood by centrifuging at 3000 rpm for 10min and were stored in refrigerator at -80°C until analysis.

Sample Pretreatment

About 500μL of patient plasma was extracted with 1mL of t-BME twice and vortexed for 1 min. After centrifugation at 2000 rpm for 10 min, the organic layer was transferred into a 10 mL beaker. The t-BME extract was evaporated to dryness, residue was reconstituted with 1mL of diluent and filtered through 0.22 μ pore size nylon membrane filters, about 20 μL of the sample was applied to HPLC analysis as per the proposed chromatographic conditions (13).

HPLC Conditions

Chromatographic separation of paclitaxel was performed using C₁₈ column (150×4.6mm i.d., 4μm particle size) with 60% acetonitrile, 40% 10mM ammonium acetate buffer solution and 0.1% formic acid as mobile phase at a flow rate of 1.0 mL/min at ambient temperature. The detection wavelength was 230 nm. Detector output integration was carried using Class VP software to determine peak areas.

Validation

The validation was performed as per ICH Q2(R1) guidelines (14).

Statistical methods

The data was statistically treated using Microsoft excel 2010. Statistical methods like relative standard deviation, percentage and coefficient of determination (r²) were used.

RESULTS

Linearity and detection limits

The limit of detection was found to be 5 ng/mL and limit of quantification that can be reliably and reproducibly measured

is 15 ng/mL. Linearity of detector response was assessed for extracted plasma standards over the range of 6-6000 ng/mL with an r^2 value of 0.9998 (Table I). For 0.5 mL aliquots of plasma samples, linearity was satisfactory between 6 and 6000 ng/mL.

Precision

Assay precision was found to be satisfactory in the concentration range of 6-6000 ng/mL. The intraday and interday precision was assessed using six replicates of three concentrations 60, 600, 6000 ng/mL and the relative standard deviation (%RSD) was found to be < 0.7% for intraday and < 3% for interday precision (Table II).

Formulation Assay

Accuracy of the method was established by quantification of paclitaxel in the pharmaceutical dosage form (Taxeleon 30 mg/5mL, Neon laboratories) in the concentration range of 6-60000 ng/mL. The results shows that the peak shape is symmetrical with a good baseline and there was strong correlation between the standard solutions and pharmaceutical dosage form solutions with respect to retention time and peak area. The assay performance data are presented in Table II.

Recovery

Recovery of paclitaxel was estimated by using spiked plasma samples of 500 μ L with standard solutions of known concentrations, extraction of plasma samples as per the sample pretreatment procedure, analyzing and comparison of peak areas of paclitaxel standard and extracted plasma samples. Recovery of paclitaxel measured in triplicates at three concentrations (60 ng, 600 ng and 6 μ g/mL) was in the range of 96-99% (Table II).

Selectivity and Specificity

Chromatograms of blank plasma, paclitaxel standard in diluent, paclitaxel extracted from patient plasma are illustrated in figure 1. The results of extraction from plasma and standard reveal a symmetrical peak shape and good baseline resolution of paclitaxel. Interference due to plasma matrix components was not observed during the analysis. Using this system, the retention time for paclitaxel was 3 min and the runtime for total analysis of each run was efficiently maintained for 5 min.

It is also observed that there is no interference of endogenous peaks, peak shape and retention time being the same for paclitaxel standard in diluent, paclitaxel formulation and extracted plasma samples.

Stability of paclitaxel

The stability of paclitaxel standard solution and extracted plasma were assessed for 6h to determine the optimum re-

quirements for processing and storage during the analysis (Table III). We found that paclitaxel was quite stable in room temperature and at 4°C for the assay duration.

Robustness

Three chromatographic conditions (Detection wavelength, Flow rate and composition of mobile phase) were altered deliberately at a range of \pm 2% and under each condition samples were analyzed to estimate the paclitaxel content. The results reveal that the values were in the acceptance limits of 98-102% with %RSD of less than 2% (Table IV).

Effect of Cremophor El on paclitaxel analysis

It is reported that the bioanalysis of paclitaxel reproducibility is negatively affected by Cr El (9). However, no such effects were observed in our study. This may be due to liquid-liquid extraction with t-BME, that nullifies the ion suppression effects caused by Cr El (15).

DISCUSSION

The limit of detection and limit of quantification of the current developed method is sensitive enough to carry out the clinical studies/pharmacokinetic studies of paclitaxel for 24 h. These are equal to the methods using sophisticated solid phase extraction (10). The value of regression coefficient $r^2=0.9998$ confirmed the linear relationship between the concentration of the drug and area of the peak. This method is precise enough to perform continuous and regular quantification of paclitaxel in human plasma. The minimal variation in %RSD for interday and intraday precision indicates the complete harmony among repeated injection, repeated analysis, intraday and interday study.

The strong correlation between the standard solutions and pharmaceutical dosage form with respect to retention time and peak area establishes the accuracy of the method. The proposed method of analysis was highly selective and specific as peaks of the analyte in spiked plasma and patient plasma were well resolved. Separation of peaks was confirmed by using blank plasma, plasma spiked with analyte and patient plasma containing analyte along with other drugs. The recovery of paclitaxel was efficient and equal to the costly SPE techniques which were used in other methods (10).

The minor deliberate changes made in various experimental parameters did not significantly affect the peak area, recovery of the analyte indicating the robustness of the developed method. The stability of paclitaxel in various conditions has been well studied. In this method stability of paclitaxel in standard solutions and extracted from plasma was assessed for 5 h both at room temp (bench-top) and under refrigeration at 4°C to establish the stability of the analyte during the process of analysis. The %RSD values shows that there were

no considerable changes in their concentration after 5 h indicating that samples are stable during analysis.

The extraction solvent used in this method t-BME can eliminate the negative influences caused by the Cr El that are reported in other methods (9). It was successfully applied for quantification of paclitaxel in human plasma and pharmaceutical dosage form.

CONCLUSION

The current developed RP-HPLC method for quantification of paclitaxel in human plasma is rapid, economic, sensitive and reproducible without negative influence of Cr El. Limit of quantification of this method was 15 ng/mL, making it applicable for clinical pharmacokinetic studies of paclitaxel. A number of methods have been described earlier for quantification of paclitaxel in various biological matrices using various techniques including HPLC. The later involve longer run times, usage of costly SPE procedures and methods to reduce the influence of CrEl. The method described here is equally sensitive yet simpler, using single solvent extraction procedure and having a short retention time of 3 min.

Mobile phase composition, sample extraction using t-BME and the use of C₁₈ (15× 4.6 cm i.d, 4 µm particle size) column have attributed to the sensitivity, precision, reproducibility and shorter retention time of the method, which makes it a very practical method to use.

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Table I: Linearity

S. No.	Concentration (ng/mL)	Retention Time (min)	Peak Area	Slope ($Y = 45.243x - 679.5$)	r^2	LOD	LOQ
1	6	3.025	187				
2	30	3.025	1795				
3	60	3.025	2853				
4	120	3.025	4299			5ng/mL	15ng/mL
5	300	3.025	12339	45.243	0.9998		
6	600	3.025	26839				
7	900	3.025	36987				
8	1200	3.025	54500				
9	6000	3.025	271041				

r^2 - Regression Coefficient, LOD- Limit of Detection, LOQ- Limit of Quantification

Table II: Assay, Precision and Recovery of paclitaxel from human plasma.

Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Assay(%) Formulation n = 3	Precision Intraday (%RSD) n = 6	Interday (%RSD) n = 6	Recovery (%) Amount (ng/mL) n = 3	
6	6.12	102.13				
60	59.13	98.56	0.3	1.6	5.80	96.73
600	598.8	99.80	0.6	2.8	596.33	99.38
6000	5970	99.5	0.02	2	5913.99	98.56
60000	61260	102.10				

%RSD- Relative Standard Deviation

Table III: Stability of paclitaxel

Time (h)	Concentration (ng/mL)	Retention time (min)	Peak Area (Bench top)	Peak Area (Refrigerator)	%RSD (Bench top)	%RSD (Refrigerator)
Stability of paclitaxel in standard stock solution						
0	60000	2.983	2668756			
1		2.983	2685985	2666899		
2		2.983	2666055	2666429		
3		2.983	2667478	2665681	0.663	0.060
4		2.983	2668765	2669276		
5		2.983	2636895	2669756		
Stability of paclitaxel in extracted plasma samples						
0	60000	2.983	2680824	2664026		
1		2.983	2692954	2659489		
2		2.983	2674790	2666269		
3		2.983	2664553	2669908	0.536	0.336
4		2.983	2655916	2651498		
5		2.983	2655869	2646749		

%RSD- Relative Standard Deviation

Table IV: Robustness of the method

Parameter	Concentration (ng/ml)	Retention Time (min)	Peak Area	% Recovery
Detection wave length – 232nm	60	3.000	2814	98.63
	600	3.000	26339	98.13
	6000	3.000	265979	98.13
Detection wave length – 232nm	60	3.000	2832	99.26
	600	3.000	27127	101.07
	6000	3.000	272862	100.67
Flow Rate – 0.8ml/min	60	3.733	2885	100.41
	600	3.733	272270	101.60
	6000	3.733	267298	98.61
Flow rate – 1.2ml/min	60	2.500	2880	100.94
	600	2.500	26390	98.32
	6000	2.500	268775	99.16
Mobile Phase ratio – 70:30	60	2.133	2859	100.21
	600	2.125	27716	102.24
	6000	2.125	272932	100.67
Mobile Phase ration – 50:50	60	5.600	2796	98.00
	600	5.600	26379	98.28
	6000	5.600	269606	99.47

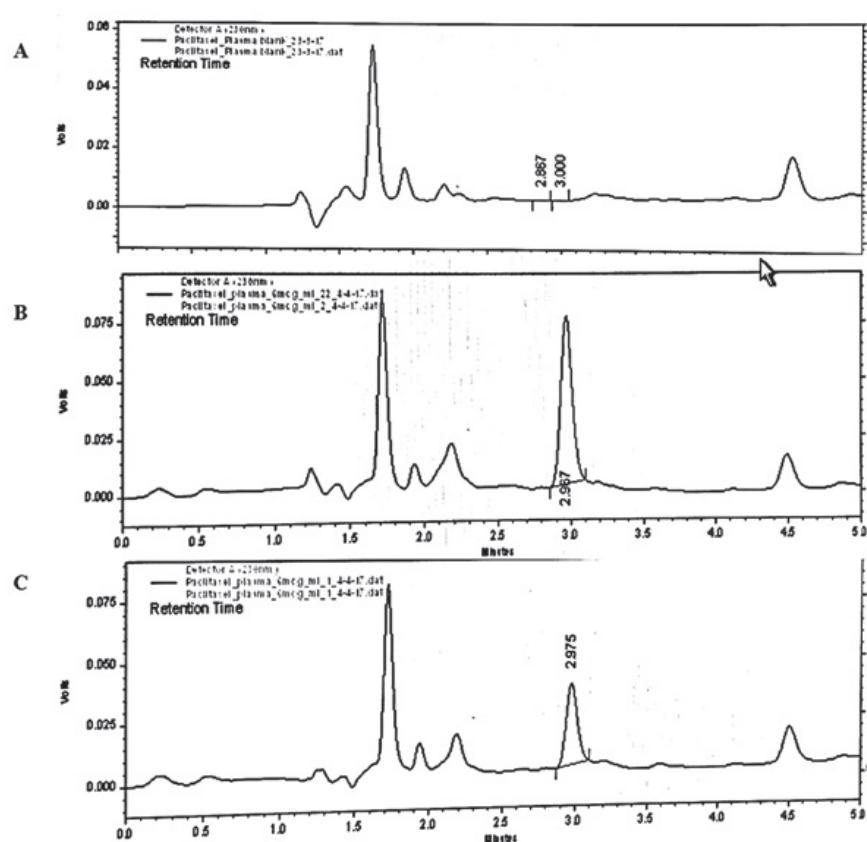


Figure 1: Chromatograms of paclitaxel; A. Chromatogram of drug free plasma, B. Chromatogram showing elution of peak with 60µg of standard paclitaxel spiked in plasma, C. Chromatogram showing elution of peak in patient plasma.